Antiviral activity of Favipiravir (T-705) against a broad range of paramyxoviruses *in vitro* and against Human Metapneumovirus in hamsters.

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Running Head: Favipiravir activity against Paramyxoviruses

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Abstract

The clinical impact of infections with respiratory viruses belonging to the family *Paramyxoviridae* argues for the development of antiviral therapies with broad-spectrum activity. Favipiravir (T-705) has demonstrated potent antiviral activity against multiple RNA virus families, and is presently in clinical evaluation for the treatment of influenza. Here, we demonstrate *in vitro* activity of T-705, against the paramyxoviruses Human Metapneumovirus (HMPV), Respiratory Syncytial virus, Human parainfluenza virus, measles virus, Newcastle Disease virus and Avian Metapneumovirus. In addition, we demonstrate activity against HMPV in hamsters. T-705 treatment inhibited replication of all paramyxoviruses tested *in vitro*, with EC_{90} values of 8 to 40 μM. Treatment of HMPV-challenged hamsters with 200 mg/kg/day resulted in 100% protection from infection of the lungs. In all treated and challenged animals, viral RNA remained detectable in the respiratory tract. The observation that T-705 treatment had a significant effect on infectious viral titers, with limited effect on viral genome titers, would be in agreement with its proposed viral mutagenesis mode of action. However, next generation sequencing of viral genomes isolated from treated and challenged hamsters did not reveal (hyper)-mutation. Polymerase activity assays revealed a specific effect of T-705 on the activity of the HMPV viral polymerase. With the reported antiviral activity of T-705 against a broad range of RNA virus families, this small molecule would be a promising broad range antiviral drug candidate to limit the viral burden of paramyxoviruses and to be evaluated for treatment of (re-) emerging viruses such as the henipahviruses.

Introduction

The family *Paramyxoviridae* contains a number of viruses causing respiratory tract illnesses in humans, which together account for a high clinical impact (1). Human Metapneumovirus (HMPV), Respiratory Syncytial Virus (RSV), and Parainfluenza virus (PIV) infections are responsible for severe acute respiratory illnesses mainly in young children, but also in immune compromised and elderly individuals, and are - together with the influenza viruses (family *Orthomyxoviridae*) - the primary viral causes of hospitalizations for severe respiratory tract disease (2-5). No licensed vaccines or effective antiviral treatments are available for these viruses. Measles virus, yet another paramyxovirus, is responsible for devastating disease for which the WHO committed to eradication with the aid of effective vaccines. However, due to suboptimal immunization levels, resurgence in infections are detected and there is no effective antiviral treatment available for measles virus infected patients (6-8). Paramyxoviruses are well known for their zoonotic potential; the Avian Pneumovirus (AMPV) is the proposed avian ancestor of HMPV and the avian Newcastle Disease Virus can cause disease – primarily conjunctivitis - in humans (9-11). Multiple novel paramyxoviruses have been detected in bats, including close relatives of human viruses (12, 13) and henipahviruses continue to cause infections in humans in Australia and Asia (14-17). No licensed vaccines or effective antiviral treatments are available for any of these viruses. The continuous burden of disease associated with human and zoonotic paramyxoviruses, argues for the development of antiviral therapies with broad-spectrum activity against all paramyxoviruses.

Favipiravir (T-705; 6-flouro-3-hydroxy-2-pyrazinecarboxamine) is a pyrazine derivative that has demonstrated potent antiviral activity against multiple RNA viruses (18, 19). Intracellular host enzymes act upon T-705, converting T-705 to its

active form T-705-4-ribofuranosyl-5-triphosphate (T-705RTP) (20). T-705RTP functions as a purine nucleotide analog that selectively inhibits the RNA-dependent RNA polymerase (RdRp) or causes lethal mutagenesis upon incorporation into the viral RNA (21-24). T-705 is presently in clinical development as an influenza virus inhibitor in Japan (New Drug Application filed) and the United States (Phase 3 clinical trial) (18). Antiviral activity has been demonstrated against a broad range of negative-stranded RNA viruses, such as members of the *Picorna-*, *Arena-*, *Bunya-*, and *Filoviridae* (25-30) and positive strand RNA viruses such as *Noro-* and *Flaviviruses* (31, 32). Here we evaluated activity of T-705 against a broad range of paramyxoviruses *in vitro* and against HMPV in hamsters.

Materials and Methods

Cells and viruses. The construction of recombinant rHMPV NL/00/01-GFP and NL/99/01-GFP and the generation of wildtype HMPV strains NL/00/17 and NL/94/01, PIV-3 (clinical isolate from 2001) and Avian Pneumovirus type C (AMPV-C) virus stocks in Vero-118 cells have been described previously (33-35). Stocks of measles virus Edmonston strain expressing GFP (MeV-Edm-GFP; a kind gift of Dr. P. Duprex) were generated in Vero-Slam cells as described before (36). The recombinant RSV A2 strain that expresses GFP (RSV-GFP) was a kind gift of Dr. M.E. Peeples and Dr. P.L. Collins (37). RSV-GFP was grown and titrated in HEp-2 cells in DMEM supplemented with 2% fetal calf serum. Influenza virus stocks (H1N1 A/Netherlands/602/09) were generated in MDCK cells in Dulbecco modified Eagle medium (DMEM; Lonza, Breda, The Netherlands) as described (38) and virus stocks of Herpes Simplex Virus (HSV)-F strain (39) were generated in Vero-118 cells in a similar way as HMPV, without addition of trypsin but with addition of 2% fetal

calf serum. Virus stocks of Newcastle Disease Virus (NDV) were generated in embryonated chicken eggs as described (40). For MeV-Edm-GFP, PIV-3, AMPV-C and HMPV, infected cells and supernatants were harvested and centrifuged 5 minutes at 300 x g and the cell-free supernatants were subsequently purified on sucrose gradients (60-30%) and aliquots were stored at –80 °C. The titer of the virus stocks was determined by end-point titration in Vero-118 (HMPV, AMPV-C, PIV-3, NDV), HEp-2 (RSV-GFP), MDCK (influenza virus) or Vero-SLAM (MeV-Edm-GFP) cells and expressed in tissue culture infectious dose 50 ($TCID_{50}$)/ml. Virus titrations were read out either by fluorescence (rHMPV NL/00/01-GFP, rHMPV NL/99/01-GFP, RSV-GFP, MeV-Edm-GFP), immune-staining (HMPV NL/94/01 and NL/00/17, AMPV-C, NDV, PIV-3), standard hemagglutination assays (influenza virus), or by reading cytopathic effects (HSV).

***In vitro* testing.** T-705 was obtained from Boc Sciences (New York, USA) and suspended in DMSO. A serial dilution of T-705 was made in infection medium with a final DMSO concentration in the cell culture supernatant <0.1%. Control samples were treated with equal amounts of DMSO as used in the treated samples. Activity of T-705 was tested against 100 $TCID_{50}$/well of RSV-GFP in HEp-2 cells, MeV-Edm-GFP in Vero-slam cells, influenza virus in MDCK cells, NDV, AMPV-C, PIV-3 and HSV in Vero-118 cells, and against 300 $TCID_{50}$/well of the four genotypes of HMPV in Vero-118 cells. Inoculations were performed in infection media as described under 'Cells and viruses' and references herein. At day 5 (HMPV), 4 (RSV-GFP, NDV, AMPV-C, PIV-3, MeV-Edm-GFP) or 2 (influenza virus and HSV) after inoculation, 100 µl of supernatant was harvested for virus titration and cells were examined under the microscope. For those viruses not expressing GFP, immunostaining preceded the

118 microscopic examination. EC_{90} values were calculated based on the dose-response
119 curves as described (41).
120
121 **Immunostaining assays.** Immunostaining for HMPV NL/17/00 and NL/94/01,
122 AMPV-C and NDV was conducted as described previously (34) (40). Similarly,
123 immunostaining for PIV-3 was conducted using the IMAGEN™
124 Immunofluorescence Test for PIV, from Oxoid (Thermo Fisher Scientific,
125 Landsmeer, The Netherlands).
126
127 **Cytotoxicity assay.** Quadruplicates of 2 x 10^4 cells per well in 96-well plates
128 (Corning) were either mock inoculated or inoculated with a 2-fold serial dilution
129 series of T-705 in infection medium, starting with a concentration of 250 μM. At time
130 point 72 hours post inoculation, cell viability was determined using the CytoTox 96
131 Non-Radioactive Cytotoxicity Assay (Promega, Leiden, the Netherlands) as described
132 (42).
133 **Polymerase assays using mini-genomes.** The construction and use of the mini-
134 genome system for polymerase assays for HMPV has been described (33, 43). The
135 mini-genome system of HMPV NL/1/00 was used to replace the CAT open reading
136 frame with that of Firefly Luciferase using standard PCR and cloning assays. As
137 control, a plasmid was used where the Firefly Luciferase expression was driven by the
138 T7-promoter (in house development). To test for the effect of T-705 on the
139 polymerase activity, 5 x 10^5 293-T cells were plated per well of a 6-well plate in
140 DMEM medium supplemented with 10% Fetal Calf Serum, 100 U ml^{-1} penicillin, 100
141 U ml^{-1} streptomycin, 2 mM L-glutamine (PSG) 1 mM sodium pyruvate (Life
142 Technologies, Bleiswijk, The Netherlands) and non-essential amino acids. Cells in

each well were transfected using the CaPO4 precipitation method (44) with 1 µg minigenome cDNA construct of HMPV NL/1/00, 50 ng pSV40-Renila-Luciferase (Promega),, 0.8 µg pCITE-N, 0.4 µg pCITE-P, 0.4 µg pCITE M2.1 and 0.4 µg pCITE-L (polymerase complex proteins of HMPV). Control cells, to quantify T-7 polymerase activity, were established by transfection with 50 ng SV40-Renila-Luciferase, pT7-Firefly-Luciferase and 1.5 µg pAR3126 (expressing a T7 RNA polymerase (45)). Transfections were done overnight and medium was refreshed the next morning with subsequent addition of serial dilutions of T-705. At 48 hours after transfection, luminescence was measured as described (45).

***In vivo* testing.** Serial dilutions of T-705 were prepared fresh daily by mixing the correct amount of compound in filtered sterilized 0.4% sodium carboxymethyl cellulose (CMC) in Milli-Q water. Six 6-week-old Syrian golden hamsters per group were orally treated with 25-50-100-150-200 mg/kg T-705 solution twice a day, during 4 days, starting 24 hours before nasal-inoculation with 1 x 10^6 $TCID_{50}$ HMPV strain NL/00/01. Throat swabs were collected at day 3 and 4 after inoculation and lungs and nasal turbinates at 4 days after inoculation for virus titration and real time RT-PCR assays.

Virus titration and real time PCR assays. Collected lungs, nasal turbinates and throat swabs were processed as described before (46). In brief, tissues were homogenized by using a Polytron homogenizer (Kinematica AG) in infection medium and supernatants were used for virus titration in Vero-118 cells. Titres were calculated $(g\ tissue)^{-1}$, with a detection limit of $10^{1.6}$ and $10^{1.2}$ $TCID_{50}$ $(g\ tissue)^{-1}$ for nasal turbinates and lungs, respectively. Viral RNA was isolated using the MagnaPure LC system with the MagnaPure LC Total nucleic acid isolation kit (Roche Diagnostics,

Almere, The Netherlands), with an elution volume of 50 µl. HMPV genome copies were detected by Taqman real-time qRT-PCR as described (47).

454-sequencing. Viral RNA was extracted from nasal turbinate and lung samples collected from hamsters, converted to cDNA and amplified by PCR using primers amplifying 3 overlapping fragments around the polymerase protein gene, covering nt. 6748-8974, nt. 8330-10170 and nt. 10158-12340 and the complete nucleoprotein open reading frame (nt. 55-1240), in total constituting 51% of the viral genome. PCR fragments for each sample were pooled in equal concentrations, and libraries were created for each sample following the manufacturer's instructions. Emulsion PCR and GS Junior 454 sequencing runs were performed according to instructions of the manufacturer (Roche). Sequence reads were sorted by bar code, trimmed at 30 nucleotides from the 3′ and 5′ ends to remove primer sequences, and the 3′ ends were further trimmed to improve quality using a Phred score of 20. Reads were aligned to reference sequence HMPV NL/00/01 (AF371337.2) using CLC Genomics software 4.6.1. The threshold for low frequency variant detection was set at 2%, with quality values $Q \geq 20$.

Animals and ethics regulation. All experiments involving animals were conducted strictly according to European guidelines (EU directive on animal testing 86/609/EEC) and Dutch legislation (Experiments on Animals Act, 1997). The experimental protocol was reviewed and approved by an independent animal experimentation ethical review committee, not affiliated with Erasmus MC (DEC consult number EMC2949).

Statistics

Statistical analysis was performed with GraphPad 6.0 (GraphPad Software).

Continuous data between groups were compared using ANOVA. If this test resulted in $P<0.05$, then pairwise Mann Whitney tests were performed.

Results

Antiviral activity of T-705 against paramyxoviruses *in vitro*

The inhibitory effect of T-705 on infection was evaluated for RSV-GFP, PIV-3, NDV, AMPV-C, MeV-Edm-GFP and prototype strains of the 4 genotypes of HMPV (rHMPV NL/00/01-GFP (A1), HMPV NL/00/17 (A2), rHMPV NL/99/01-GFP (B1) and NL/94/01 (B2)). The addition of high concentrations of T-705 (up to 1600 μM) 24 hours prior to inoculation had no or little effect on cell viability, as measured by cytotoxicity assay. Microscopic examination of the cells revealed that with addition of increasing T-705 concentrations the number of infected cells decreased for all viruses tested (Figure 1). For MeV-Edm-GFP, addition of 62.5 μM resulted in the absence of typical MeV-Edm-GFP induced plaques. For most of the other viruses, a decrease in infection efficiency of the cells was observed starting at a concentration of 125 μM T-705. Only for RSV-GFP, higher concentrations of the compound were necessary to decrease infection of HEp-2 cells (Figure 1).

In a next experiment, as a preparation of the *in vivo* experiments, we set out to mimic the effect of T-705 in a pre-exposure and post-exposure setting of virus infection *in vitro*. Cells were treated at 3 time points: 24 hours prior to-, simultaneous with -, or 24 hours after inoculation. Influenza virus and HSV were taken as sensitive and insensitive controls, respectively. Furuta *et al*. reported HSV to be insensitive to T-705 treatment, with EC_{50} values >625 μM. In our study, we observed some virus yield reduction when T-705 was administered 24 hours prior to- and simultaneous with infection, with EC_{90} values of 230 and 540 μM, respectively (Table 1). As these values are significant higher than those obtained for the sensitive influenza virus (P=

0.0286 and P=0.0286 for treatment prior to-and simultaneous with inoculation; Mann Whitney P<0.05) and treatment after infection with HSV had no inhibitory effect on virus release (EC_{90}>1500 μM), our data confirm the insensitivity of HSV to T-705 treatment (Figure 2). Using plaque reduction assays, Furuta *et al* have reported EC_{50} values for influenza virus ranging from 0.083 - 2.9 μM (19). Here, we obtained EC_{90} values of 1.5 μM and 2.0 μM, for treatment prior to- and simultaneous with infection, respectively. Taken in account that the employed assays were different, plaque reduction assays vs. yield reduction and EC_{50} vs. EC_{90}, these values are in a similar micromolar range, which confirms the activity of our batch of T-705 against the influenza virus. However, the influenza virus was significant less sensitive for treatment after infection, compared to treatment prior to- or simultaneous with infection (P=0.03 and 0.03, respectively; Mann Whitney P<0.05) (Table 1; Figure 2). Of all paramyxoviruses tested, the MeV-Edm-GFP virus was the most sensitive virus with EC_{90} values of 8.6, 9.7 and 13 μM for treatment prior to-, simultaneous with-, and after inoculation, respectively (Table 1). For treatment prior to- or simultaneous with infection, these values are significant higher than those for influenza virus (P=0.03 and P=0.03; Mann Whitney P<0.05), although the EC_{90} values for MeV-Edm-GFP virus are still in the micromolar range. The MeV-Edm-GFP virus and influenza virus are similarly sensitive to T-705 treatment at 24 hours after infection, with EC_{90} values of 57 and 13 μM for influenza virus and the MeV-Edm-GFP virus, respectively (P=0.2; Mann Whitney P<0.05). Together, these data show that MeV-Edm-GFP is sensitive to treatment with T-705 in a similar micromolar range as that is effective against influenza virus (Figure 2).

The four genotypes of HMPV were sensitive to treatment in the same micromolar rage as the MeV-Edm-GFP virus, with EC_{90} values ranging from 11-26 μM, 12-34

241 μM and 22-43 μM for treatment prior to-, simultaneous with-, and after infection,
242 respectively (Table 1). In general, serotype A strains showed to be slightly less
243 sensitive to T-705 compared to serotype B strains. Although no significant differences
244 were observed between the four strains for treatment before infection (P= 0.09;
245 ANOVA P<0.05) or after infection (P= 0.07; ANOVA P<0.05), significant
246 differences between the four HMPVs were observed for treatment simultaneous with
247 infection (P= 0.003; ANOVA P<0.05) (Figure 2). This was mainly due to the
248 significant higher EC_{90} value of NL/00/17 (34 μM) compared to those of the type B
249 viruses (15 and 12 μM, for NL/99/01 and NL/94/01, respectively) for this time point
250 of treatment (P=0.0286 and 0.0286, respectively; Mann Whitney P<0.05). HMPV
251 strains NL/00/01, NL/99/01 and NL/94/01 were equally sensitive as MeV-Edm-GFP
252 for treatment prior to inoculation (P=0.34, 0.48 and 0.52, respectively; Mann Whitney
253 P<0.05). Only NL/00/17 was less sensitive than MeV-Edm-GFP for this treatment
254 (P=0.03; Mann Whitney P<0.05). All four HMPVs, as well as MeV-Edm-GFP, were
255 equally sensitive to treatment after infection as the influenza virus (P=0.14; ANOVA
256 P<0.05) (Figure 2). These results obtained for four genotypes of HMPV demonstrate
257 that this virus is sensitive to treatment in a similar micromolar range as that is
258 effective against the influenza virus and the MeV-Edm-GFP virus.
259 T-705 also demonstrated an inhibitory effect on virus release for APMV-C, NDV,
260 PIV-3 and RSV (Table 1). No significant differences were observed between these
261 four viruses for the three treatments (P=0.35, 0.08 and 0.37 for prior to-, simultaneous
262 and after infection, respectively; ANOVAP<0.05). However, the EC_{90} values of these
263 four viruses were all significant higher than those for HMPV strain NL/00/01 (P=
264 0.01, 0.002, 0.02 for prior to-, simultaneous with- and after infection, respectively;

ANOVAP<0.05), strain NL/99/01 (P= 0.002, 0.0003, 0.02), strain NL/94/01 (P= 0.0015, <0.0001, 0.01), but not than those of HMPV NL/00/17 (P= 0.07, 0.02, 0.08). All together, these data show that T-705 has an antiviral effect, in the micromolar range, against all these paramyxoviruses viruses with higher EC_{90} values for AMPV-C, NDV, PIV-3 and RSV compared to HMPV and MeV-Edm-GFP virus.

In vivo antiviral activity of T-705 against HMPV

To test the *in vivo* activity of T-705, an established animal model for HMPV infections was employed. Syrian golden hamsters were treated with 50-100-150-200-400 mg/kg/d during 4 days, starting 24 hours before nasal-inoculation with 10^6 $TCID_{50}$ HMPV strain NL/00/01 (type A1). During this experiment the animals did not show any signs of illness or weight loss.

Real time RT-PCR assays conducted on throat swabs collected at day 3 and 4 after inoculation and qRT-PCR assays conducted on nasal turbinate and lung samples collected at day 4 after inoculation, revealed the presence of viral genomes in these samples at all given concentrations (Figure 3A and B, top panels). However, the number of viral genomes in the throat swabs and nasal turbinates decreased significantly with administration of increasing concentrations of T-705: in the throat swabs starting at a dose of 100 mg/kg/d at day 3 (P=0.026; Mann Whitney P<0.05), at a dose of 200 mg/kg/d at day 4 (P=0.01; Mann Whitney P<0.05), and in the nasal turbinates at a dose of 200 mg/kg/d (P=0.026; Mann Whitney P<0.05). T-705 treatment did not result in a significant decrease in viral genome titers in the lungs (P= 0.2; ANOVA P<0.05), although the titers did decrease with increasing concentrations of T-705 (Figure 3B, top left panel). Virus titration of the throat swab samples revealed levels of infectious virus below the limit of detection in 3 (day 3) or 4 (day4)

290 of the 6 animals treated at a dose of 100 mg/kg/d. In general, infectious virus titers in
291 the throat swabs decreased significant starting at a dose of 100 mg/kg/d (P=0.0022;
292 Mann Whitney P<0.05) (Figure 3A, lower panels), and in the nasal turbinates and
293 lungs at 200 mg/kg/d (P=0.015 and P=0.022, for nasal turbinates and lungs,
294 respectively; Mann Whitney P<0.05) (Figure 3B, lower levels). Most importantly, at a
295 dose of 200 mg/kg/d 3 out of 6 animals displayed levels of infectious virus below the
296 limit of detection in their lungs and at a dose of 400 mg/kg/d none of the animals had
297 levels of infectious virus above the limit of detection in their lungs (Figure 3B, lower
298 right panel).

299 Although replicating virus could not be detected in the lungs of animals treated with
300 400 mg/kg/d, viral genome titers only declined minimal. These findings of decreased
301 infectious virus titers in samples from treated animals with only limited decrease in
302 viral genome titers, resemble the findings reported for the influenza virus. In that
303 study, the infectious virus load in treated samples decreased disproportionately
304 compared with the RNA copy number. The authors demonstrated that T-705 induced
305 hypermutation of the viral genome, which explained the decrease in viral titers with
306 equal titers of viral genome copies in samples treated with T-705 (24). To investigate
307 this possibility for the mechanism of action of T-705 against HMPV, samples (nasal
308 turbinates and lungs) of untreated animals and of high-dose treated animals were
309 subjected to next generation sequencing (see Table 2). To this end nt. 6748-12340 of
310 the viral genome (the L open reading frame) for two animals each (H013, H014:
311 untreated; H044,H045: treated) were subjected to 454-deepsequencing, using 3
312 overlapping PCR fragments. Overall, this region was covered by 6892 to 17447 reads
313 (Figure 4A) and analysis for single nucleotide polymorphisms (SNPs) revealed the
314 presence of 22 SNPs in the viral genomes isolated from treated animals that were

315 also present in the viral genomes retrieved from untreated hamsters (Supplemental
316 table S1). This analysis also revealed the presence of approximately 25 reads with T
317 to C hypermutation, but these were only detected in the sample isolated from the nasal
318 turbinate of one treated hamster (H043-blue circles in figure 4A around nt.8000).
319 These hypermutated reads were absent in the lung sample of the same hamster and in
320 the samples from the other treated hamster (H044). These hypermutated reads most
321 likely represent (hypermutated) DIs that occur during HMPV infection, as previously
322 reported (48), and, based on the absence in the other (treated) samples, are not a result
323 of T-705 treatment. The T-C hypermutation detected in these reads were included in
324 calculation of the number of SNPs for figure 4C, which explains the wide error-bars.
325 Additional SNPs were detected that were present in untreated samples and absent in
326 treated samples or the other way around. In the NT samples of untreated animals, 9
327 (H013) and 10 SNPs (H014) were detected and in these lung samples 4 (H013) and 5
328 (H014) SNPs were detected that were absent in samples from treated animals, all of
329 them were present in < 10% of the reads (Supplemental table S1). In the NT of treated
330 animals 4 SNPs (in both H043 and H044) and in the lungs 16 (H043) and 4 (H044)
331 SNPs were detected that were absent in the untreated samples. Overall the number of
332 SNPs detected in this part of the genome did not differ significantly between the
333 treated and untreated samples, even when the 25 hypermutated reads detected in the
334 NT of H043 were included (Figure 4C). To obtain more evidence and to avoid the
335 detection of DIs, the nucleoprotein gene of the genomes isolated from the same
336 animals, and including a third animal per group (H015, H041) was also subjected to
337 deep sequencing. To this end, one PCR fragment covering the complete ORF was
338 analyzed, and deep sequencing resulted in coverage of this region by

22655 to 49335 reads (Figure 4B). Similar to the results obtained for the polymerase protein ORF, SNPs detected in the viral genomes isolated from treated animals were also present in the samples obtained from untreated samples, and were present in only less than 10% of the reads (Supplemental Table S2). There were no significant differences between the number of SNPs detected in the nucleoprotein ORF in the samples obtained for treated and untreated samples (Figure 4C). Together, next generation sequencing of approximately 50% of the viral genomes of samples obtained from treated and untreated animals did not reveal significant differences in SNPs detected in treated and untreated samples (Figure 4C), which indicates that T-705 did not induced specific mutations in these parts of the viral genome.

To investigate whether T-705 had a direct effect on the activity of the polymerase proteins of HMPV, as described for those of influenza virus and chikungunya virus (18, 49), the inhibitory effect of T-705 on polymerase complex activity was evaluated with a mini-genome system. Addition of DMSO alone (data not shown), or increasing concentrations of T-705 had no inhibitory effect on the expression of the constitutive expressed Renilla-Luciferase, or on expression of a T7-promoter driven Firefly-luciferase expression plasmid (Figure 5A, left panel). In contrast, addition of increasing concentrations of T-705 to the mini-genome system of HMPV, expressing firefly-luciferase, demonstrated an inhibitory effect of T-705 on the HMPV polymerase activity (Figure 5, black bars). Addition of 80 μM reduced the activity with 50%, compared to untreated samples, while addition of 800 μM T-705 significantly reduced the luciferase activity to below 10% ($P=0.002$; T-test $P<0.05$). These data demonstrate that for HMPV the mechanism of action of T-705 is, at least

363 partially, directed against the polymerase activity and not the induction of lethal
364 mutagenesis of viral genomes.
365

366 ## Discussion

367 T-705 has been shown to be effective against important respiratory viruses, such as
368 influenza viruses and rhinoviruses. In this study we demonstrated activity of T-705
369 against important human respiratory viruses of the family *Paramyxoviridae*: HMPV,
370 RSV, PIV and the measles virus. The EC_{90} values detected *in vitro* were in the low
371 micromolar range (on average below 10 µg/ml), similar as observed for influenza
372 virus (EC_{50} 0.013-0.46 ug/ml) (19), bunyaviruses (EC_{50} 5-30 µg/ml), arenaviruses
373 (0.7-1.2 µg/ml) and picornaviruses (4.8-23 µg/ml) (25). Using plaque reduction
374 assays, Furuta *et al.* (19) reported EC_{50} values of 260 µM for RSV. In our yield
375 reduction assays, we found EC_{90} values of 36 to 70 µM for the different treatment
376 regimens. In both studies, lab adapted strains of RSV were used. The activity of T-
377 705 against RSV might be higher against wild type strains, which would be
378 worthwhile to investigate.
379 In addition, differences between observed values may be related to the use of different
380 assays, such as plaque reduction assays vs reduction in virus yields, and presentation
381 of the data in EC_{50} or EC_{90}, but in general these values are sufficiently low to be
382 translated for *in vivo* use.
383 Our results also demonstrate activity against the avian paramyxoviruses AMPV-C and
384 NDV. Although these viruses do not cause substantial disease in humans,
385 paramyxoviruses are well known for their zoonotic potential. The activity against
386 these avian viruses reveals that T-705 has activity against a broad range of

paramyxoviruses and therefore it is possible that this compound would also be active against henipahviruses.

To translate *in vitro* activity into *in vivo* activity we employed the established hamster model for HMPV infections (50). In this model, T-705 treatment decreased virus replication in the throat , nasal turbinates and lungs of the inoculated animals starting at a dose of 200 mg/kg/d and at dose of 400 mg/kg/d none of the animals had detectable levels of infectious virus in their lungs. Previous vaccination studies for HMPV have also resulted in protection of the lungs but not the nasal turbinates upon challenge. The primary goal of treatment of respiratory virus infections is prevention of serious lower respiratory tract illnesses, and in this *in vivo* model treatment with T-705 protected against infection of the lungs. The dose of 200-400 mg/kg/d is similar to doses used in other animal models for other viruses (19). In addition, these doses are within the range as reported for influenza virus infection, for which the compound is now approved in Japan and evaluated in clinical trials elsewhere (www.clinicaltrials.gov).

Our *in vitro* pre- and post-exposure experiments revealed that T-705 is more effective when treatment starts 24 h before infection or during infection. Especially for the influenza virus and HSV, which are both fast replicating viruses, late addition of the compound was less effective. Although EC_{90} values were higher at late addition for the paramyxoviruses, values for all three treatments were roughly in the same range. As T-705 needs to be metabolized into its active metabolite T-705-4-ribofuranosyl-5-monophosphate (T-705RMP) and then to T-705-4-ribofuranosyl-5-triphosphate T-705RMP, it requires several hours to reach an effective concentration (21, 51). This could explain why the compound is less effective against fast replicating viruses when treatment occurs post infection. Comparison of the results obtained for inhibition of

infection (Figure 2) and for virus yield reduction (Figure 3) demonstrated that T-705 had more effect on virus yield than on virus infection, as lower concentrations of compound were needed to reduce the virus yield than to inhibit virus infection.

The mechanism of action of T-705 remains a topic of investigations. Both direct inhibition of the polymerase as well as induction of lethal mutagenesis have been described (23, 24, 49, 52). In the T-705 treated hamsters, inoculated with HMPV, viral genomes were detected in all animals even when treated at high doses. This presence did not always correlate with the presence of infectious virus, suggestive for an effect of the compound on the formation of infectious virus. This is further supported by our data showing that treatment had more effect on virus yields than on viral infection (Figure 3 vs. Figure 2). Deep sequencing of approximately half of the viral genome of samples obtained from untreated and treated animals, did not reveal any T-705 induced mutations in these parts of the viral genome. As no materials were left to sequence other parts of the genome, we cannot rule out the presence of mutations in other parts of the genome that could explain the mechanism of action of T-705 against HMPV. However, the mutations detected in the afore mentioned studies on influenza virus, norovirus and chikungunya virus were induced during passaging of the virus under pressure of high concentrations of T-705. Whether these types of mutations are also occurring in *in vivo* situations remains unsure. In a mouse model for persistent norovirus infections, where the animals were treated for 8 weeks, an average of 2.9 fold increase in mutation frequencies were found in virus samples obtained from T-705-treated animals compared to control animals (52). However, in this case the virus is under pressure for a prolonged period of time (8 weeks), which cannot be compared with the 4-day treatment in the HMPV hamster model.

436 We did detect T-C hypermutation in genomes obtained from the NT of one treated
437 animal. These were all detected in approximately 25 (hypermutated) reads. These type
438 or reads with T-C hypermutation have been detected previously during HMPV
439 infections *in vitro* (48) and are therefore most likely not a result of T-705 treatment.
440 This is, as far as we known, the first time that HMPV DIs are detected during *in vivo*
441 infection, and is of interest for future investigations.

442 The absence of (hyper)mutations in the nucleoprotein gene, the region where the
443 qRT-PCR primers align, proofs that the increase in CT values in T-705 treated
444 samples (figure 3) are not due to changed sensitivity of the qRT-PCR assay but due to
445 activity of T-705.

446 Using mini-genome assays to investigate the effect of T-705 on the polymerase
447 activity of HMPV, we showed that adding T-705 to this assay resulted in a significant
448 decrease in the polymerase activity. Currently, there are two hypotheses for the
449 mechanism of action of T-705: a) the induction of lethal mutagenesis and/or (b) chain
450 termination by the incorporation of favipiravir-RMP into the nascent RNA strand
451 (23). Our data indicate that T-705 does not induce lethal mutagenesis on HMPV
452 genomes, but does have a direct effect on the HMPV polymerase. This is in line with
453 the activity against the influenza virus polymerase RNA-dependent RNA polymerase
454 as reported by Kiso *et al*. (53) and with data reported for the chikungunya virus,
455 where a single mutation in the polymerase protein was sufficient to make the virus
456 resistant to treatment (49). Although it is possible that T-705 interferes with viral
457 morphogenesis at a late stage of the infectious cycle, T-705 is a nucleotide analog and
458 its mechanism of action is thought to be related to the selective inhibition of viral
459 RNA-dependent RNA polymerase of influenza virus and many other RNA viruses
460 (21, 23). Time-of-addition experiments have demonstrated that T-705 inhibits the

early to intermediate stage of viral replication of arenaviruses and noroviruses (32, 54). However, the exact mechanism of action against paramyxoviruses still remains to be elucidated.

In conclusion, we show that T-705 has antiviral activity against a broad panel of paramyxoviruses, both human and avian. With the reported antiviral activity of T-705 against a broad range of RNA virus families, this small molecule would be a promising broad range antiviral drug candidate to limit the viral burden of respiratory viruses and to be evaluated for treatment of (re-) emerging viruses such as the henipahviruses. Importantly, elucidation of the mechanism of action of T-705 against all these viruses would allow the design of novel compounds that employ similar mechanisms of action, which could help in the development of antiviral treatment option for a broad spectrum of respiratory viruses.

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677 ## Figure Legends

678

679 **FIGURE 1: *In vitro* testing for T-705 activity against paramyxoviruses.** Cells were
680 treated with serial dilutions of T-705 starting 24 hours prior to inoculation with the
681 respective virus. At 5 days post inoculation, GFP expressing viruses (RSV-GFP,
682 MeV-Edm-GFP, rHMPV strains NL/00/01-GFP and NL/99/01-GFP) were examined
683 directly under a microscope, and the other viruses were examined after virus specific
684 immunofluorescence staining as described in the materials and method section.

685

686 **FIGURE 2: Virus yield reduction assays.** *In vitro* activity of T-705 against
687 paramyxoviruses as well as HSV and influenza virus as controls. Cells were treated
688 starting 24 hours prior to inoculation (black bars), simultaneous with inoculation
689 (dark grey bars) or 24 hours after inoculation (light grey bars). At 2-5 days after
690 inoculation, infectious virus yield in the supernatants were determined and EC_{90}
691 values calculated. Different batches of T-705 were tested and averages and ranges are
692 given of two independent experiments, both conducted in duplicate. Statistical
693 analysis were done with One way Anova and Mann Whitney tests, for which results
694 are described. *=$P<0.05$; Anova or Mann-Whitney test.

695

696 **FIGURE 3: *In vivo* activity of T-705 against HMPV.** Six 6-week old hamsters per
697 group were orally treated with 50-100-200-300-400 mg/kg.day T-705, during 4 days,
698 starting 24 hours prior to nasal-inoculation with 10^6 $TCID_{50}$ HMPV strain NL/1/00.
699 Throat swabs were collected at day 3 and 4, nasal turbinates and lungs at 4 days p.i.
700 A) Ct-values obtained with real-time RT-PCR assays (top panels) and viral titers

(bottom panels) for throat swabs collected at day 3 (left panels) and day 4 (right panels). B) Number of HMPV genomes as determined by qRT-PCR (top panels) and viral titers (bottom panels) per gram nasal turbinate (left panels) or lung (right panels). Circles indicate values of individual animals, and black line the average value of 6 animals. *=P<0.05; **=P<0.01; Mann-Whitney test.

FIGURE 4: Detection of mutations in the A) Polymerase protein and B) the nucleoprotein gene of HMPV by 454-sequencing. Graphic representation of coverage and viral variant analysis in nasal turbinates (NT) and lung tissue (L) for hamsters untreated (H013;H014;H15)) or treated with 300 (H041) or 400 mg/kg/day T-705 (H044,H045). Lines indicate the coverage over the target region (left y-axis). **Blue diamonds, black** dots and red triangles indicate variants detected per sample. Position of the dots on the x-axis indicates the position in the sequence where variation was found. Position on the right y-axis indicates the amount of variation at that position. C) the number of SNPs detected in the polymerase protein region, the nucleoprotein region (NT) and combined for both regions of the viral genomes isolated from nasal turbinates (NT) and lungs from treated and untreated samples. Black spots indicate individual SNP numbers per animals, the black (horizontal) line the average for all animals and standard deviations are indicated. Using Mann-Whitney (P<0.05), no significant differences in number of SNPs between untreated and treated animals was found.

FIGURE 5: Effect of T-705 on polymerase activity. 293T cells transfected with: A) T7-Firefly-Luciferase and transient expressed control (SV40-Renilla-Luciferase) or B) the HMPV mini-genome system expressing Firefly-Luciferase and the transient expressed control (SV40-Renilla-Luciferase) were treated with serial dilutions of T-

705 at 18 hours after transfection. Luminescence was read at 48 hours after transfection as described in Materials and Methods. Black bars: Firefly-Luciferase activity; Grey bars: Renilla Luciferase activity. C) Percentage of luminescence compared to the untreated samples (luminesce of untreated samples are set at 100%). Graphs represent values obtained in one, representative, experiment conducted in duplo. *=P<0.05, T-test.

Table 1:

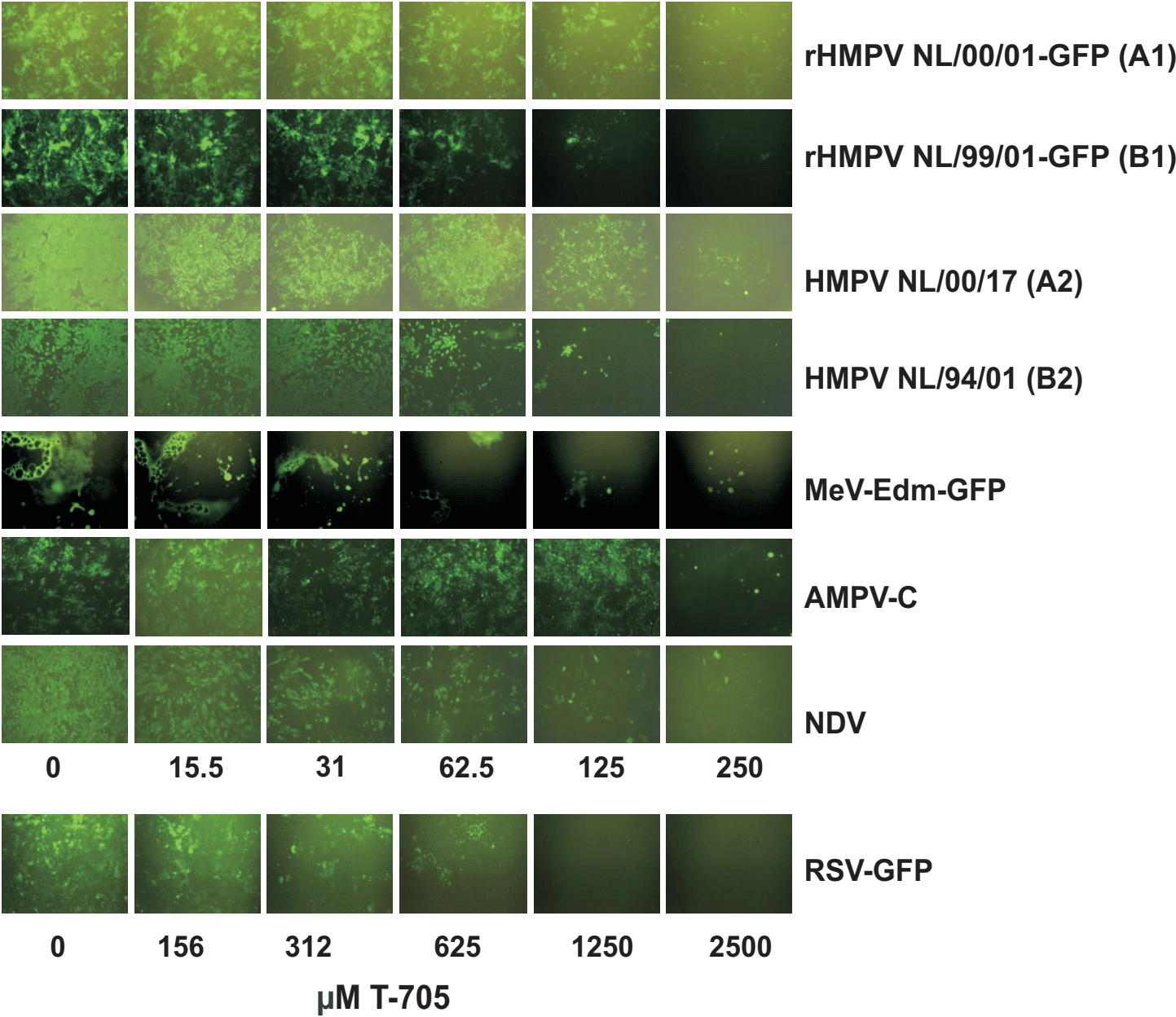
In vitro activity of T-705 against paramyxoviruses as well as HSV and influenza virus as controls. Cells were treated starting 24 hours prior to-, simultaneous with - or 24 hours after inoculation. At 2-5 days after inoculation, infectious virus yield in the supernatants were determined and EC_{90} values calculated. Different batches of T-705 were tested and averages and ranges (st.dev) are given of two independent experiments, both conducted in duplicate.

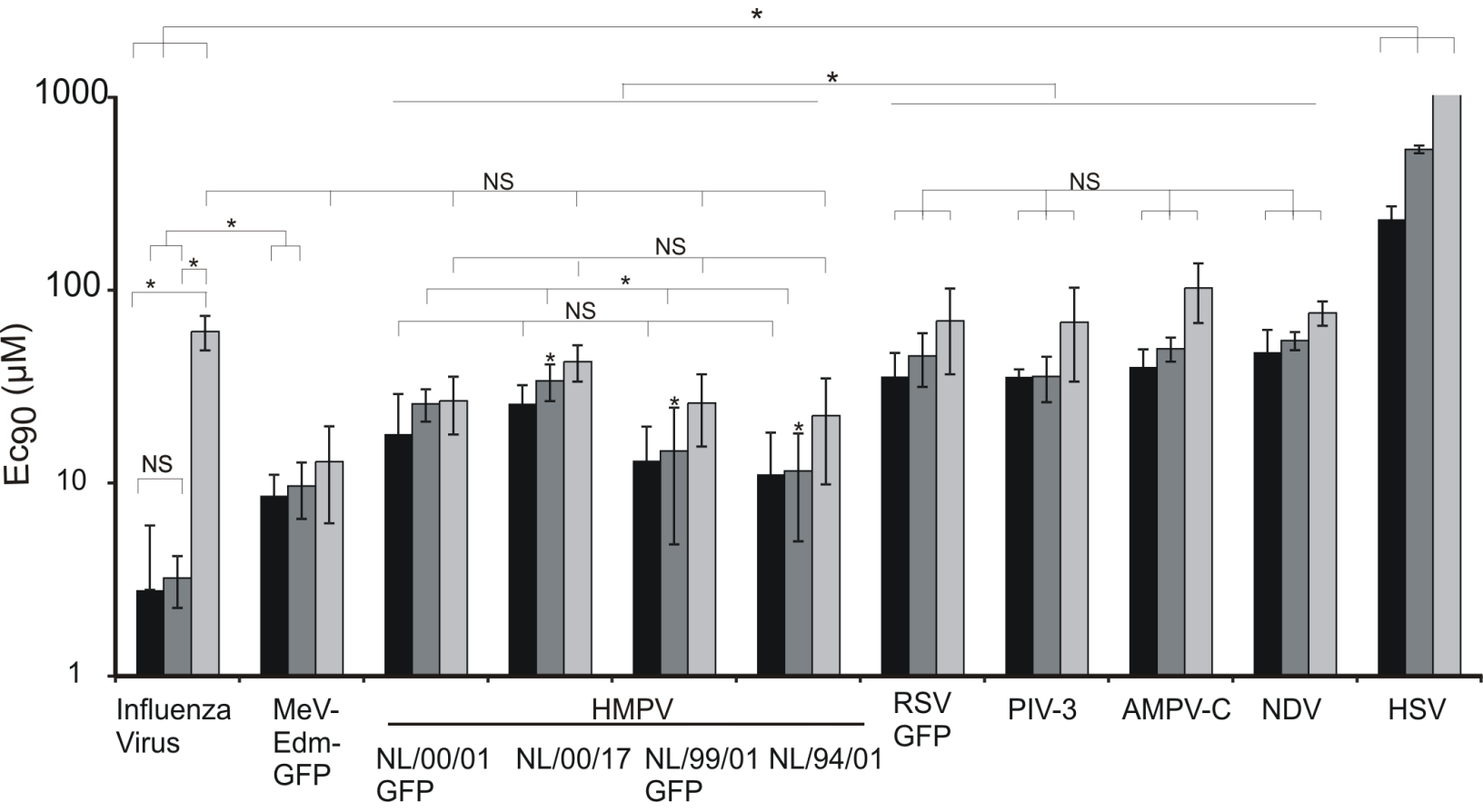
		Prior to -		**Simultaneous with-**		**After inoculation**	
		EC_{90} (µM)	**st. dev**	**EC_{90} (µM)**	**st. dev**	**EC_{90} (µM)**	**st. dev**
Influenzavirus		1.5	1.3	2	2.2	57	35
MeV-Edm-GFP		9	2	10	3	13	7
HMPV A1	NL/00/01	18	11	26	5	27	9
HMPV A2	NL/00/17	26	6	34	7	43	9
HMPV B1	Nl/99/01	13	7	15	10	26	11
HMPV B2	NL/94/01	11	7	12	7	22	13
RSV-GFP		36	12	46	14	69	33
PIV-3		36	3	36	9	68	35
AMPV-C		40	9	50	7	103	35
NDV		48	15	55	6	76	11
HSV		233	39	538	257	>1500	nd

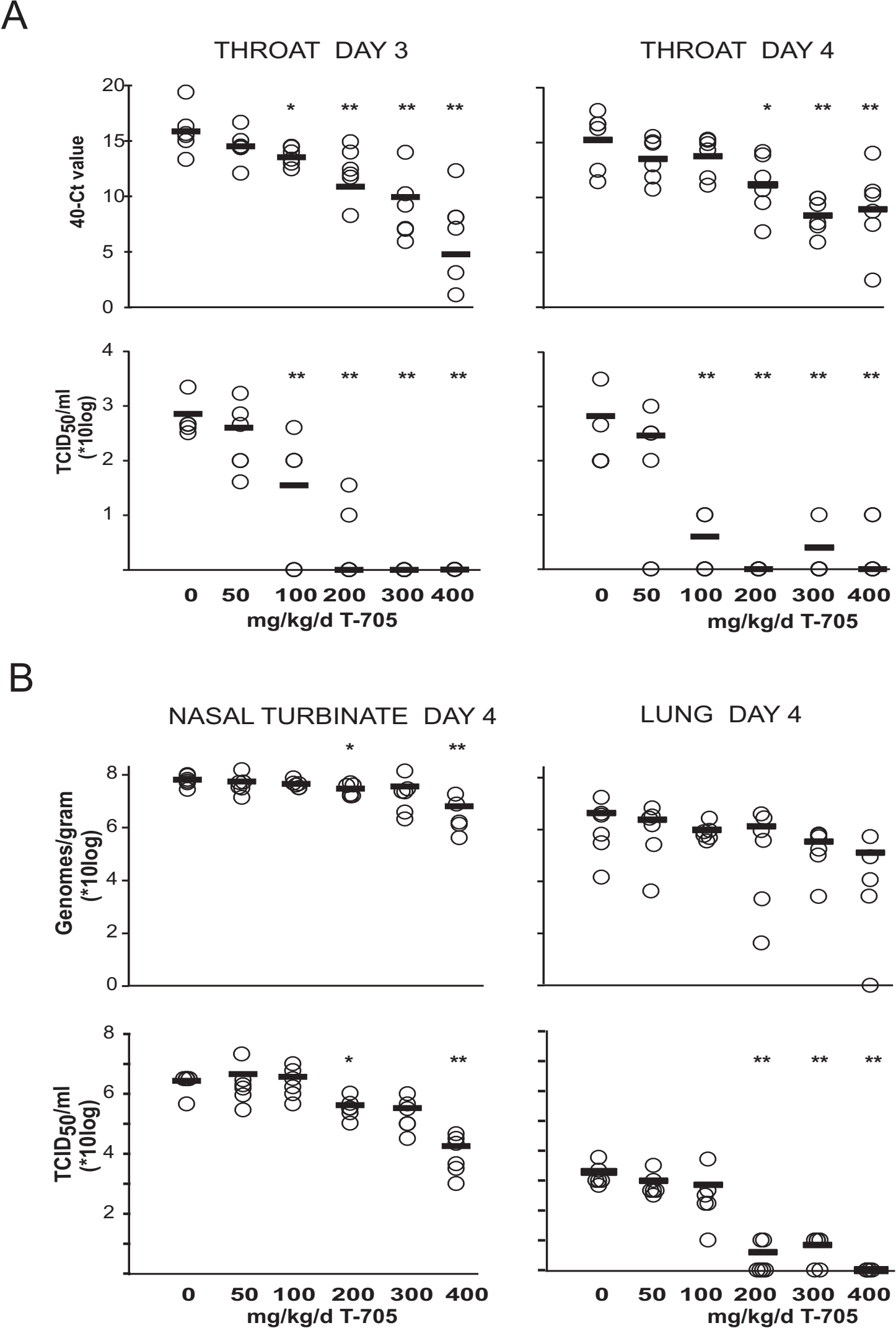
Table 2

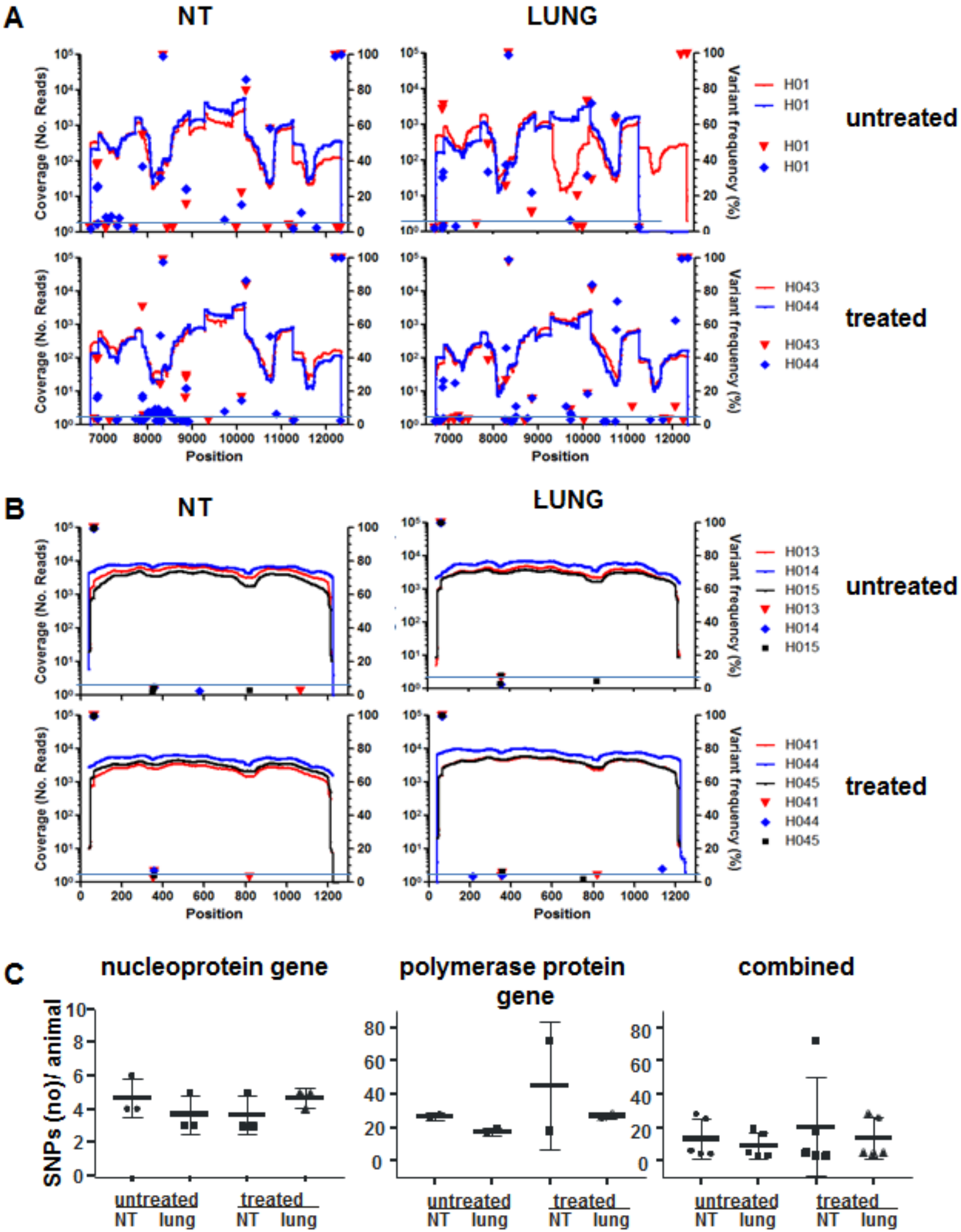
Information on treatment, Ct values obtained in qRT-PCR and virus titration for hamster samples selected for deep sequencing. NT=nasal turbinate

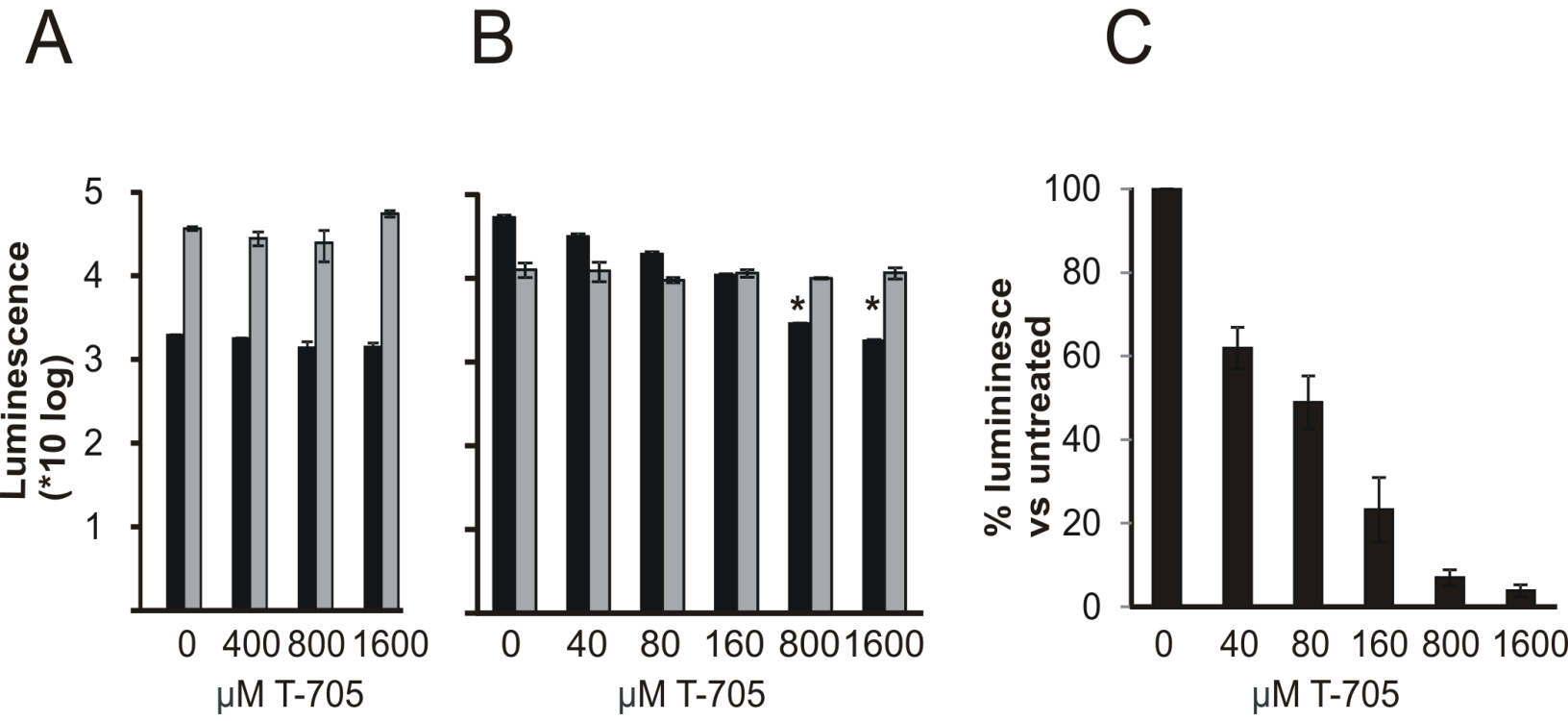
Animal	**T-705 (mg/kg/day)**	**CT Lung**	**Titer lung ($TCID_{50}$/ gram)**	**CT NT**	**Titer NT genomes/ gram**
H013	0	18	$7,2x10^3$	17	9,39E+07
H014	0	25	$3,4x10^3$	19	4,60E+07
H015	0	21	$3,4x10^3$	19	2,60E+07
H041	300	25	0	20	2,09E+07
H044	400	23	0	21	1,87E+07
H045	400	24	0	21	7,25E+06


rHMPV NL/00/01-GFP (A1)
rHMPV NL/99/01-GFP (B1)
HMPV NL/00/17 (A2)
HMPV NL/94/01 (B2)
MeV-Edm-GFP
AMPV-C
NDV
0
15.5
31
62.5
125
250
RSV-GFP
0
156
312
625
1250
2500
µM T-705


1000
100
10
1
Ec90 (μM)
NS
*
Influenza Virus
MeV-Edm-GFP
HMPV
NL/00/01 GFP
NL/00/17
NL/99/01 GFP
NL/94/01
RSV GFP
PIV-3
AMPV-C
NDV
HSV


A
THROAT DAY 3
THROAT DAY 4
40-Ct value
TCID50/ml (*10log)
0 50 100 200 300 400
mg/kg/d T-705
B
NASAL TURBINATE DAY 4
LUNG DAY 4
Genomes/gram (*10log)
TCID50/ml (*10log)
0 50 100 200 300 400
mg/kg/d T-705


A
NT
LUNG
Coverage (No. Reads)
Variant frequency (%)
Position
H01
H01
H01
H01
untreated
H043
H044
H043
H044
treated
B
NT
LUNG
H013
H014
H015
H013
H014
H015
untreated
H041
H044
H045
H041
H044
H045
treated
C
nucleoprotein gene
polymerase protein gene
combined
SNPs (no)/ animal
untreated
treated
NT
lung


A
B
C
Luminescence (*10 log)
5
4
3
2
1
0
400
800
1600
μM T-705
0
40
80
160
800
1600
μM T-705
*
*
% lumininesce vs untreated
100
80
60
40
20
0
0
40
80
160
800
1600
μM T-705